**List of changes made by the authors in the revised manuscript JoVE52792R1**

*Changes in the revised manuscript (RM) are typed in green (underscored) or red.*

**Changes suggested by the Science Editor**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The authors followed this advice.

2. Throughout the text, dissect would sound better than "tear" for much of the animal work. On line 156 (RM) “Tear off” has been replaced by “Dissect”.  
  
3. JoVE reference format requires that DOIs are included, when available, for all references listed in the article. The authors have done maximal efforts to obtain DOIs of the references. This was, however, not possible for a number of old references: even consultation of the original papers and books did not yield DOIs.

**Changes suggested by the Reviewers.**  
  
**Reviewer #1:**

Protocol 1.1 (prep PHFs): Using a 9-day-old fertilized chick egg, "completing the incubation 4 days before the start…." is not clear and should be better explained. This sentence has been rephrased in the revised manuscript: “Complete this incubation by a date that allows subsequent preparation of PHFs during 4 days (*e.g.* on a Thursday,) and final confrontation with tumor cell aggregates (*e.g.* on the next Monday)

Protocol 1.4: Is the MEM-Rega 3 medium without FBS? The MEM-Rega 3 medium contains 5% FBS. This is specified in the revised manuscript.

Protocol 1.6: Is "fresh culture medium" meant to be the MEM-Rega 3 medium? With or without FBS? This medium is indeed MEM-Rega 3 medium containing 5% FBS. This is specified in the revised manuscript.  
  
Figure 2: either arrows pointing towards or insets enlarging the "paired aggregates" should be useful, as they constitute only a minor part of the picture. In the revised figure the 8 PHFs are individually indicated by arrows.

Figure 3: arrows pointing towards the differently-sized Erlenmeyers and indicating their content, and arrows showing the CO2/air gas flow direction would be helpful. Is this device home-made? On the revised figure numbers are applied whose sequence indicates the gas flow in the suspension culture Erlenmeyer flasks. and are explained in the legend. The authors also specified in the revised figure legend that the device was home-made.

Protocol 6. Concerning the toxicity assessment: in addition to explant outgrowth, probably classical immunohistochemical tests for cell viability/proliferation and cell death (eg apoptosis) could be used? In a note added to section 6 the use of Ki67 immunohistochemistry (for cell proliferation) and of TUNEL assay (for apoptosis) are suggested as complementary techniques to the explant assay for assessing toxicity.

Protocol 7. Concerning the growth assessment: the volume of the PHF does not increase but remains stable or decreases so one takes into account only the growth of the "confronting" cells? If so, this could be specified in the text. The comment of the reviewer is pertinent: during incubation solitary PHFs tend to decrease their volume. In a note added to section 7 the prominent impact of the confronting tumor cells on the growth of the confronting cultures is mentioned.

Figure 5 and corresponding text: the predictive QSAR model and its "classes" should be better explained or rephrased. In the revised manuscript the legend of this figure is rewritten to allow its interpretation independently from the main text:

*“External validation of a predictive QSAR model (artificial neural network) for the activity of small molecules in the CHI assay. The output of this model is the anti-invasive activity class of a compound. Four such classes have been defined, representing the lowest concentration at which a molecule exerts anti-invasive activity (i.e. invasion grade I or II) in the CHI assay: class 4 (active down to 1 µM), class 3 (10 µM), class 2 (100 µM) and class 1 (no anti-invasive activity at concentrations as high as 100 µM). The depicted confusion matrix compares predicted and experimentally determined anti-invasive activity classes for the compounds of the validation set. The validation set contains 46 compounds, the training set 93. Model predictions are based solely on descriptors calculated from molecular structure, and can thus be obtained for hypothetical compounds. This way, synthetic efforts can be focused on molecules with promising in silico activity.”*   
  
**Reviewer #2:**

The protocol description is not very clear at present. For example, the precise step where the heart fragments and cancer cells are mixed is not indicated at present. The authors could provide a flowchart of key steps. In the revised manuscript a schematic flowchart of the key assay steps is added as figure 1.   
  
Models of organ culture tend to display large batch to batch variations. Some illustration of technical/biological reproducibility of assays will be useful. This comment of the reviewer is correct, and we believe that we addressed the reproducibility of the assay in the paragraph from line 479 to line 487 in the RM:

*The data show the robustness of the chick heart invasion assay, since the correlation between*

*predicted and experimental results was valid over 15 years, and confirmed in a recent (unpublished) prediction study with different polyphenolics. The confusion matrix presented in Figure 6 summarizes the weakness and the strength of the assay graphically: it gives a rough expression of the accuracy and the reproducibility. The interpretation of this graph should take into account the biological variability of living organ cultures, and the semi- quantitative score of the invasion results.*

*[Place Figure 6 here]*   
  
Figure 4 shows selected pictures representing different stages of the assay. If possible, this figure should include a pa. rallel panel of cells with different invasiveness (a non-invasive cell line, or the invasive cell line treated with a drug that inhibits its invasiveness). A demonstration of the utility of the technique presented here to capture "differences" in cell invasiveness is key to the potential utility of this platform for the wider community. We fear that this comment is based on a misunderstanding. Figure 4 does not show the chronological progression of invasion by malignant tumor cells in the assay. Rather, it illustrates the final results obtained from cells with different invasiveness. These range from grades 0, I, II (coined non-invasion) to grades III,IV (invasion).   
  
**Reviewer #3:**  
  
There is a lack of figures actually showing how the heart is removed, fragmented and cultured to yield the host spheroids. We agree with the reviewer that the treatment of the heart can be better illustrated than described. However, actually we do not dispose of macrographs of the consecutive dissection steps, and, considering the limited revision time allotted, we were not able to organize a new experiment for making new pictures. We propose to take into account this reviewer’s comment when it could come to video registration of this technique.

Histological figures presented are too small and should be marked with arrows to distinguish the key zones. Labels are missing to indicate what is the tumor being studied as this is a working example. In the revised manuscript the figure is enlarged, and heart or tumor zones are indicated with characters (“H” and “T” respectively)   
  
Figure 5 is not informative without the additional information behind the analysis. In the RM the legend of this figure is rewritten to allow its interpretation independently from the main text:

*“External validation of a predictive QSAR model (artificial neural network) for the activity of small molecules in the CHI assay. The output of this model is the anti-invasive activity class of a compound. Four such classes have been defined, representing the lowest concentration at which a molecule exerts anti-invasive activity (i.e. invasion grade I or II) in the CHI assay: class 4 (active down to 1 µM), class 3 (10 µM), class 2 (100 µM) and class 1 (no anti-invasive activity at concentrations as high as 100 µM). The depicted confusion matrix compares predicted and experimentally determined anti-invasive activity classes for the compounds of the validation set. The validation set contains 46 compounds, the training set 93. Model predictions are based solely on descriptors calculated from molecular structure, and can thus be obtained for hypothetical compounds. This way, synthetic efforts can be focused on molecules with promising in silico activity.”*    
  
What is bothersome in the protocol is the use of toxic reagents both in the fixative and antibody preservation. There are good substitutes, and the commercial firms have moved beyond use of sodium azide. The authors should offer non-toxic alternatives that work just as well. In the “caution” paragraph concerning the fixation procedure (lines 258 to 259) an alternative procedure is mentioned with 4% formaldehyde in phosphate –buffered saline. In the “caution” paragraph concerning azide (line 357) the use of 0.01% thiomersal is mentioned as an alternative preservative.   
  
1.7 'ventricular'-typo. This is corrected in the reversed version.  
  
1.9 What is the rationale for this step? The rationale for this suspension culture step is to obtain spheroidal heart tissue fragments suitable for subsequent confrontation with tumor cell aggregates. This rationale is added to the revised manuscript.  
  
**Reviewer #4:**

This experiment has been done on heart tissue that was stripped from its pericardium. What is the purpose of this step? Doesn't it diminish the significance of this assay as opposed to what happen in some types of lung or esophageal cancers that directly invade the heart along with its pericardium? The comment is considered as correct by the authors. So, the assay may indeed not be a relevant model for heart invasion by lung or esophageal cancers. For practical reasons, however, we prepare precultured heart fragments (PHFs) surrounded by fibroblastic cells, since the initial amount of pericardial mesothelium is theoretically not sufficiently present to literally cover homogeneously all PHFs needed for an experiment. The mesothelium-free myocardium-rich PHFs are meant as a general substrate to assess invasion of multiple cell types.   
  
In the text, it is mentioned that the same type of assay has been described before but from heart fragments taken from other species, or using other organs. What are the advantages or disadvantages of this particular organ? And the species? It should be discussed in the text. The choice of heart over other organ fragments was based on the spontaneous rhythmic pulsations of many of the PHFs. These pulsations are used as indicators of possible heart toxicity of test drugs in the culture medium. The authors preferred avian embryos, because they can easily be dissected from the sterile content of the egg. The rationale these choices are added to the discussion section of the RM (lines 112 to 115).

It should be described for the reader how a particular inhibitor of invasion is added to this assay? Drugs are generally delivered to the culture medium at the moment when the confronting PHF/tumor aggregate pairs are transferred to small Erlenmeyer flasks (step 2.7). This information is added in lines 465 to 478 in the RM.

The rate of variability of this technique should also be mentioned especially that it is a laborious technique that would require several days (30) to perform. This comment of the reviewer is correct, and we believe that we addressed the reproducibility of the assay in the paragraph from line 479 to line 487:

*The data show the robustness of the chick heart invasion assay, since the correlation between*

*predicted and experimental results was valid over 15 years, and confirmed in a recent (unpublished) prediction study with different polyphenolics. The confusion matrix presented in Figure 6 summarizes the weakness and the strength of the assay graphically: it gives a rough expression of the accuracy and the reproducibility. The interpretation of this graph should take into account the biological variability of living organ cultures, and the semi- quantitative score of the invasion results.*

*[Place Figure 6 here]*